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Humoral and Cell-Mediated Immune Responses in Humans to the NSP4 Enterotoxin of Rotavirus

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Rotavirus nonstructural protein NSP4 has recently been suggested to function as a viral enterotoxin and play a role in the pathophysiological mechanism whereby rotaviruses induce diarrhea. The ability of rotavirus NSP4 to stimulate a humoral immune response was examined in naturally infected children and adults, rotavirus vaccinated children, as well as a cellular immune response in adults. In this study, 10 of 10 naturally infected children and 9 of 10 rotavirus-vaccinated children showed a weak humoral IgG immune response to recombinant NSP4 (rNSP4) and/or a synthetic peptide corresponding to residues 114–134 of NSP4. Modest serum IgG antibody responses were detected in 20 of 20 adults. A cellular immune response to rNSP4 and/or $NSP4_{114–134}$ were detected in 8 of 10 adults measured either as a T-cell proliferative response (7 of 10), an increased production of IL-2 (6 of 10), or increased production of interferon-γ (8 of 10). These results indicate that NSP4 induces a humoral immune response in humans and show for the first time that NSP4 stimulates a cellular immune response, possibly including cytolytic T-cells. ***J. Med. Virol. 59:369–377, 1999***.

KEY WORDS: NSP4; enterotoxin; toxic peptide, B- and T-cell responses; human

INTRODUCTION

Rotaviruses are the most important cause of severe diarrheal disease in infants and young children [Kapikian and Chanock, 1990]. The immune mechanisms controlling and preventing rotavirus disease in humans are incompletely understood despite significant research efforts and development of an oral vaccine [Ward, 1996; Vesikari, 1997]. It is generally thought that the two outer capsid proteins VP4 and VP7 play a critical role in protective immunity by stimulating production of neutralizing antibodies [Chiba et al., 1986; O'Ryan et al., 1994]. While serotype-specific protection mediated by antibodies directed against the outer capsid proteins may be one mechanism for protection, such a correlate for protection has been difficult to demonstrate in humans [Hjelt et al., 1987; Ward et al., 1992; Ward, 1996]. Instead, vaccine trials with bovine and rhesus rotavirus strains have suggested that serotype cross-reactive immunity plays an important role [Vesikari et al., 1983; Clark et al., 1988; Bernstein et al., 1990; Vesikari, 1996]. Increasing evidence also suggests that viral proteins that lack a capacity to stimulate the production of neutralizing antibodies are fully capable of inducing a protective immune response. For example, the rotavirus inner capsid VP6 protein has been shown to induce protective immunity in mice after DNA vaccination and virus-like particles containing VP6 on their outer surface also induce protective immunity in mice [Chen et al., 1997; O'Neal et al., 1997, 1998]. In addition, a monoclonal antibody directed at VP6 protected adult SCID mice from rotavirus infection [Burns et al., 1996].

Limited efforts have focused on the role of nonstructural proteins in the induction of protective immunity to rotavirus. The protective potential of antibodies directed against nonstructural proteins has recently emerged from studies of flavivirus [Schlesinger et al., 1990; Jacobs, 1993; Hall et al., 1996], hepatitis C virus

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[Diepolder et al., 1995] and alphavirus [Gorrell et al., 1997]. NSP4, a nonstructural protein of rotavirus, functions as an intracellular receptor during subviral particle morphogenesis [Au et al., 1989]. NSP4 has also been proposed to function as a viral enterotoxin, based on its capacity to induce diarrhea in infant mice, to induce chloride secretion from intestinal mucosa [Ball et al., 1996; Tian et al., 1996] and to mobilize intracellular Ca^{2+} in insect and human intestinal cells [Tian et al., 1994, 1995; Zeng et al., 1996; Dong et al., 1997]. Two recent studies have also associated mutations in the NSP4 gene with virulence [Kirkwood et al., 1996; Zhang et al., 1998], while in other studies such an association has not been observed [Horie et al., 1997; Ward et al., 1997]. The fact that antibodies against NSP4 are capable of reducing diarrhea in infant mice when challenged with infectious virus [Ball et al., 1996] may indicate that rotavirus diarrhea in humans also could be modulated by immune responses to NSP4. Previous studies on the humoral immune response against various rotavirus proteins provide only limited information concerning NSP4 in humans [Svensson et al., 1987a,b; Richardsson et al., 1993], and no previous study has reported on cellular immune responses in humans to NSP4.

MATERIAL AND METHODS

Samples

Sera from the following patient groups were obtained for humoral immune response studies: convalescent sera collected at 20–26 days postinfection from 10 Swedish children (aged 9–22 months) who had a natural rotavirus infection, pre- and postvaccination sera from fourteen Finnish children (aged 2–5 months) who had received three oral doses of the tetravalent RRV vaccine [Joensuu et al., 1997], and sera from 10 Nicaraguan and 10 Swedish healthy adults (aged 28–58 years). Serum samples were stored at –20°C until examination. Whole blood was collected in EDTA tubes for analysis of cell-mediated immune responses, from the 10 Swedish adults and peripheral blood mononuclear cells (PBMC) was purified on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient and used immediately. Informed consent was obtained from the participants or their parents in the different study groups. The guidelines for human experimentation of the U.S. Department of Health and Human services were followed and the study was approved by the Ethical Committees for Human Studies at the three different centers involved.

Recombinant VP6 and NSP4

Recombinant NSP4 (SA11) and VP6 (SA11) were produced in baculovirus-infected Sf9 cells and purified as previously described [Ball et al., 1996; Tian et al., 1996; Zeng et al., 1996; Dong et al., 1997]. For NSP4, Sf9 cells were infected with pAC461/SA11-10 and harvested at 4 days postinfection in TNM-FH (Hink's) media containing 10% fetal calf serum (FCS). The cells were lysed with 10 mM Tris-HCl pH 8.1 buffer containing 2% sodium deoxycholate. NSP4 was first semipurified by fast protein liquid chromatography (FPLC), using a quaternary methylamine (QMA) anion-exchange column (Waters, Milford, MA) and eluted with a linear NaCl gradient. The NSP4-rich fractions were pooled and further purified using an agarose immunoaffinity column, onto which purified rabbit IgG against NSP4 had been immobilized. The bound NSP4 was eluted with 0.1 M Tris-HCl, pH 2.8 buffer, then dialyzed extensively against 50 mM NH_4HCO_3 and lyophilized. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining showed that the rNSP4 protein was approximately 90% pure. For VP6, Sf9 cells were infected with pAC461/SA11-6, and VP6 released into the medium was harvested at 6 days postinfection in serum-free SF 900II SFM (GIBCO, Grand Island, N.Y.). The medium containing VP6 was clarified by centrifugation, and VP6 in the clarified supernatant was pelleted through a 35% sucrose cushion by centrifugation in a Beckman SW28 rotor for 90 min at 25,000 rpm. VP6 oligomers in the resulting pellet were banded by isopycnic centrifugation in a Beckman SW50.1 rotor for 18 hr at 35,000 rpm. A band containing VP6 was collected by side puncture, dialyzed extensively against 50 mM NH_4HCO_3, and lyophilized. SDS-PAGE and silver staining showed that the protein was >95% pure, and unboiled VP6 exhibited the mobility of trimeric VP6.

Synthetic Peptides

A peptide consisting of rotavirus NSP4 amino acid (aa) residues 114–134 (DKLTTREIEQVELLKRIYDKL) was synthesized in an automated peptide synthesizer (SyRo; Multisyntech, Bochum, Germany) by using solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) methodology. Amino acids (Millipore, Sundbyberg, Sweden) were coupled as Fmoc-protected amino acids to an Fmoc resin. The Fmoc groups were deprotected by piperidine. Cleavage of the peptides from the resin and deprotection of the side chain groups were performed using trifluoroacetic acid, including different sets of scavangers depending on the amino acids in the peptide. After cleavage, the peptides were precipitated with ether, dissolved in 1% acetic acid, and lyophilized. The peptides were purified by high-pressure liquid chromatography (HPLC). Two pools of six 20-mer synthetic peptides representing the complete Nef regulatory protein (aa 1–205) of HIV [Hinkula et al., 1997] were produced with the same methodology and were utilized as controls in the T-cell proliferation assay and cytokine-release assays.

IgA and IgG ELISA

Briefly, polyvinyl microtiter plates with high binding capacity (Costar, Cambridge, MA) were coated with 100 μl of recombinant NSP4 (2.5 μg/ml) or 100 μl of VP6 (2.5 μg/ml) or with synthetic peptides (10 μg/ml) of $NSP4_{114–134}$ or gp41 (JB4C) from the HIV virus [Broliden et al., 1991] in 0.1 M carbonate-bicarbonate buffer (pH 9.6) overnight at +4°C. Plates were then

blocked with 1% bovine serum albumin-phosphate-buffered saline (BSA-PBS) for 30 min at 37°C, and serum samples (serial twofold dilutions starting at 1:100) were added and incubated for 90 min at 37°C for the IgG enzyme-linked immunosorbent assay (ELISA), and overnight at +4°C for the IgA ELISA. As conjugate, a hoseradish peroxidase (HRP)-conjugated rabbit-anti-human IgG (Dako, Copenhagen) diluted 1:20,000 or HRP-conjugated rabbit-anti-human IgA (Dako, Copenhagen, Denmark) diluted 1 10,000 was added for 60 min at 37°C. The reaction was developed using 3,3′,5,5′, tetramethylbenzidine (ICN Biochemicals, Cleveland, OH) at 0.1 mg/ml diluted in 0.1 M Na-acetate buffer (pH 6.0) with 0.002% H_2O_2, and the reaction was stopped with 2 M H_2SO_4. Optical density was determined at 450 nm. Antibody titers were defined as the reciprocal of the highest dilution giving a net OD value > 2 SD above the OD reading of a negative sample.

T-Cell Proliferation

The T-cell proliferative activity to recombinant NSP4 and a synthetic peptide corresponding to residues 114–134 of NSP4 were examined in 10 healthy Swedish adults essentially as previously described for HIV [Mathiesen et al., 1989; Wahren et al., 1989]. In a 96-well tissue culture plate (Costar) 1.5×10^5 peripheral blood mononuclear cells (PBMC)/well were added to triplicate wells for every test antigen. The antigens were tested in two concentrations of 1 μg/well and 0.1 μg/well. Culture medium and human immunodeficiency virus (HIV)-specific nef synthetic peptides [Hinkula et al., 1997] served as negative controls, while phytohemagglutinin (PHA; Orion Diagnostica, Trosa, Sweden) and tetanus toxoid (SBL, Stockholm, Sweden) served as positive controls. PBMCs and antigen were cultivated in RPMI 1640 media supplemented with 4 mM L-glutamine, 50 IU/ml penicillin and 50 mg/ml streptomycin and 10% sera from ABO donors for 6 days at 37°C in 5% CO_2. At day 6, cells were pulsed with ^{3}H-labeled thymidine (0.5 Ci/50 l) for 4 hr at 37°C and immediately harvested in a Wallac cell harvester (Wallac, Turku, Finland). Thymidine incorporation was measured using a β-counter. Background proliferation in medium alone was within the range of 200–1,100 cpm. The stimulation index (SI) was calculated by dividing the mean cpm-values of the antigen-stimulated cells with the mean cpm of the medium control cells. A >5-fold SI with the rNSP4 and a >2-fold SI with the synthetic peptide were considered positive [Wahren et al., 1994; Hinkula et al., 1997].

Secretion of Interleukin-2 and Interferon-γ In Vitro

Interleukin-2. Supernatants were collected from antigen-stimulated PBMCs and tested by an interleukin-2 (IL-2)-dependent cytotoxic T-lymphoblastic (CTLL-2) cell line bioassay [Deacock et al., 1992]. A standard with rIL-2 (Amersham Int. PTC, Amersham, UK) was used to determine the level of IL-2 expression in the supernatants. Blood mononuclear cells were cultured for 48 hr with the antigens (rNSP4, $NSP4_{114–134}$ synthetic peptide, tetanus toxoid, PHA, and two pools of peptides representing the complete nef protein of HIV [Hinkula et al., 1997], as described for the PBMC proliferation assay. Cell-free medium supernatants were harvested and IL-2 content was quantitated based on the ability to support CTLL-2 cell proliferation [Deacock et al., 1992]. A total of 100 μl of harvested supernatant or rIL-2 standard was added per test well and 5,000 CTLL-2 cells added per well. Plates were incubated for 20–22 hrat 37°C before being pulsed with ^{3}H-labeled thymidine (1 μCi/well) for 4 hr. Cells were harvested in a Wallac cell harvester and the thymidine incorporation was measured in a β-counter. A positive response was defined as a stimulation inducing release of >0.1 IU/ml IL-2.

Interferon-γ. Supernatants were collected from PBMCs stimulated for 60 hr with antigen and tested in an interferon-γ (IFN-γ)-specific ELISA (Endogen, USA). A positive result was defined as a release >0.2 pg/ml.

Computer HLA Peptide Motif Search

To investigate whether the $NSP4_{114–134}$ peptide harbors T-cell epitopes with the capacity to induce a cytolytic T-cell response a computer HLA peptide motif search (Taylor R. BIMAS/CBEL/DCRT/NIH) was carried out.

Statistical Analysis

Antibody titers within a group of individuals were compared by the Wilcoxon signed ranks test. Antibody titers between groups were compared by the Wilcoxon rank sum test.

RESULTS

Humoral Immune Responses to NSP4 in Naturally Infected Swedish Children

All convalescent sera collected from the 10 naturally infected Swedish children were found to contain IgG antibodies against rNSP4 (GMT 324 SEM ± 186) and rVP6 (GMT 800 SEM ± 421) (Fig. 1). Several (4 of 10) children had IgG titers to rNSP4 only slightly above the cutoff level (>100). The IgG antibody levels were, as illustrated in Figure 1, significantly lower to rNSP4 than to rVP6 ($P < 0.005$). Six of 10 rNSP4-positive sera also recognized the $NSP4_{114–134}$ peptide (GMT 504 SEM ± 122 for the positive sera) (Table I). None of the sera contained detectable IgA antibody titers to recombinant NSP4 or VP6.

Humoral Immune Responses to NSP4 in Rotavirus Vaccinated Finnish Children

Pre- and postvaccination sera were obtained from 14 Finnish children vaccinated with the oral rhesus-human reassortant tetravalent (RRV-TV) vaccine [Joensuu et al., 1997] and were divided into two groups (Table II). The first group contained nine children who were protected against subsequent rotavirus infection during two rotavirus epidemic seasons, and the second group contained five children who developed rotavirus-

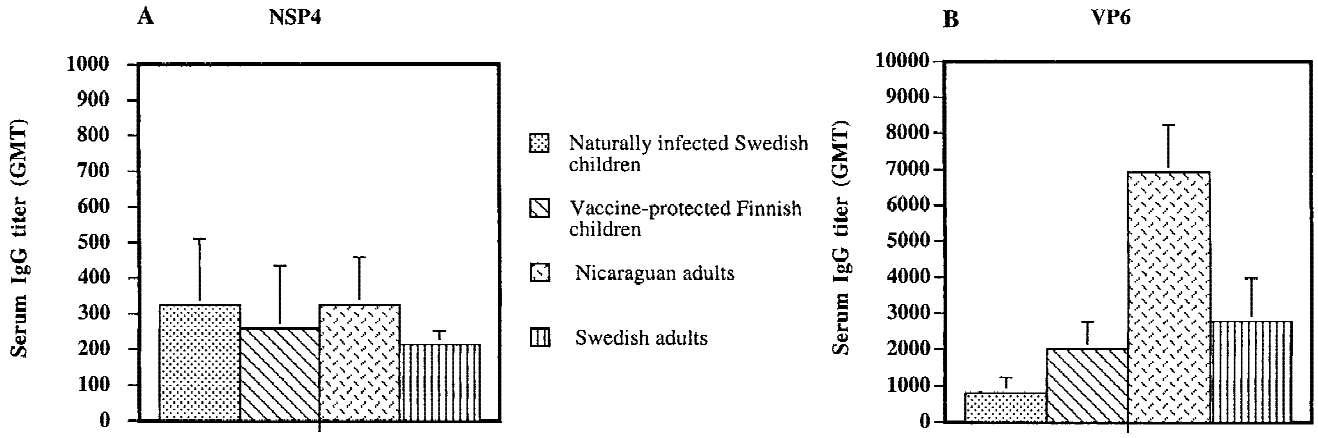

Fig. 1. Serum IgG antibody titers to rNSP4 **(A)** and rVP6 **(B)** in vaccinated and naturally infected individuals, as measured by enzyme-linked immunosorbent assay (ELISA).

TABLE I. IgG Antibody Titers to a Synthetic Peptide Corresponding to Residues 114–134 of NSP4 in Sera From Vaccinated and Rotavirus-Infected Individuals as Measured by ELISA

Individuals	Naturally infected Swedish children	Vaccinated Finnish children	Swedish adults	Nicaraguan adults
1	<100	100[a]	<100	200[a]
2	<100	<100	<100	<100
3	400[a]	<100	<100	<100
4	<100	<100	<100	<100
5	800[a]	100[a]	<100	100[a]
6	100[a]	<100	<100	<100
7	800[a]	<100	200[a]	<100
8	800[a]	800[a]	<100	<100
9	<100	200[a]	<100	<100
10	800[a]	100[a,c]	<100	<100
11[d]		<100[b]		
11[d]		800[a,c]		
12		200[a,b]		
13		<100[b]		
14		100[a,c]		

[a]Positive values.
[b]Sample taken postvaccination but before development of rotavirus-induced diarrhea.
[c]Sample taken postvaccination and after development of rotavirus-induced diarrhea.
[d]Same individual.

associated diarrhea after completion of the immunization schedule. The nine protected children (1–9, Table II) all had serum IgG antibodies to rVP6 in their postvaccination sera and all but one had IgG antibodies to rNSP4. The serum antibody titers to rNSP4 (GMT 259 SEM ± 176 for the positive sera) were significantly lower than to rVP6 (2,015 SEM ± 759) ($P < 0.005$) (Fig. 1). In 4 of 9 postvaccination sera from protected individuals (nos. 1, 5, 8, 9, Table I), an IgG response to the $NSP4_{114–134}$ peptide was noted (GMT 200 SEM ± 168 for the positive samples). A total of 11 of 14 children showed low titers against rVP6 already in the prevaccination sera. A possible explanation for this is the presence of residual maternal antibodies, as the samples were obtained upon enrollment in the study at 2–4 months of age. Serum IgA antibodies (titer >100) were found in 8 of 9 children against rVP6, but no child had detectable serum IgA antibodies against rNSP4 (data not shown).

From three of the five nonprotected children (nos. 11, 12, 13, Table II), sera were available postvaccination, but before development of rotavirus-induced diarrhea. Only one of these children (no. 12) had serum IgG antibodies to rNSP4 as well as the $NSP4_{114–134}$ peptide (Table I) and, although all three children had IgG antibodies to rVP6 (Table II), their IgG anti-VP6 titers (GMT 400 SEM ± 176) were lower as compared with the vaccine-protected (GMT 2,015 SEM ± 759) and lower as compared with the naturally infected (GMT 800 SEM ± 421) children. Unfortunately, the low number of sera does not permit any statistical analysis. Sera were also available after a natural rotavirus infection from three of the five nonprotected and vaccinated-children (nos. 10, 11, 14, Table II). All these children had IgG anti-rVP6 (GMT 5,080 SEM ± 3,200) and anti-rNSP4 (GMT 158 SEM ± 100) antibodies. All three children also had detectable IgG responses to the $NSP4_{114–134}$ peptide (Table I). Again, the low number of sera does not permit statistical analysis.

Humoral Immune Responses to NSP4 in Nicaraguan and Swedish Adults

Antibody responses to recombinant NSP4 and VP6 were examined in 10 sera collected from Nicaraguan adults. All sera were shown to contain IgG antibodies to rNSP4 (GMT 324 SEM ± 135) and rVP6 (GMT 6 912 SEM ± 1 318) (Fig. 1). Serum IgG antibodies to the $NSP4_{114–134}$ peptide were detected in only 2 of 10 Nicaraguan adults (Table I). IgA antibodies to rNSP4 were detected in 3 of 10 of these individuals and to rVP6 in 7 of 10 (data not shown). Sera and PBMC collected from 10 Swedish adults were also examined for humoral and cellular (see below) immune responses to NSP4. All individuals had IgG antibody titers to rNSP4 (GMT 214 SEM ± 37) and to rVP6 (GMT 2 786 SEM ± 1202) (Fig. 1). Serum IgG antibodies to the $NSP4_{114–134}$ pep-

TABLE II. Serum IgG Titers in Pre- and Postvaccination Sera to rNSP4 and rVP6 in Rotavirus-Vaccinated Finnish Children, as Measured by ELISA

	rNSP4		rVP6	
Children	Prevaccination	Postvaccination	Prevaccination	Postvaccination
Protected				
1	100[a]	200[a]	200[a]	6,400[a]
2	100[a]	100[a]	800[a]	6,400[a]
3	100[a]	100[a]	800[a]	3,200[a]
4	<100	200[a]	400[a]	800[a]
5	<100	400[a]	800[a]	1,600[a]
6	200[a]	400[a]	<100	800[a]
7	<100	200[a]	400[a]	400[a]
8	200[a]	1,600[a]	800[a]	3,200[a]
9	<100	<100	800[a]	3,200[a]
Not protected				
10	100[a]	100[a,b]	800[a]	12,800[a,b]
11[d]	<100	<100[c]	<100	200[a,c]
11[d]	—	400[a,b]	—	3,200[a,b]
12	<100	400[a,c]	800[a]	800[a,c]
13	<100	<100[c]	<100	400[a,c]
14	<100	100[a,b]	800[a]	3,200[a,b]

[a]Positive values.
[b]Serum taken postvaccination and after development of rotavirus-induced diarrhea.
[c]Serum taken postvaccination, but before development of rotavirus-induced diarrhea.
[d]Same individual.

tide were noted in only 1 of 10 Swedish adults (Table I). IgA antibodies to rNSP4 were detected in 4 of 10 Swedish adults and to rVP6 in 7 of 10 Swedish adults. The IgA anti-rNSP4 titers were only 100 in all four individuals (data not shown).

Cell-Mediated Immune Responses to NSP4 in Swedish Adults

To examine whether NSP4 has the capacity to stimulate a cellular immune response after a natural rotavirus infection, PBMC were isolated from 10 Swedish adults described above and first tested by a T-cell proliferative assay as described [Wahren et al., 1989]. Recombinant NSP4 antigen was found to induce a T-cell proliferative response in 4 of 10 individuals (median SI 6.8) (Table III). Using a synthetic peptide $NSP4_{114–134}$ a T-cell proliferative response was found in 5 of 10 individuals (median SI 10.7). Only one individual was found to be positive with both rNSP4 and the $NSP_{114–134}$ peptide. None of the nef peptides or culture media alone showed positive proliferation.

To examine whether a natural rotavirus infection could induce a Th1-like response and subsequently a possible CTL response against NSP4, supernatants from antigen-stimulated PBMCs were tested for IL-2 release by an IL-2-dependent CTLL-2 cell line bioassay [Deacock et al., 1992]. Briefly, PBMCs were cultured for 48 hr with the following antigens; rNSP4, $NSP4_{114–134}$ peptide, tetanus toxoid, PHA and two pools of peptides covering the complete nef protein of HIV [Hinkula et al., 1997]. Cell-free medium was harvested and IL-2 content was quantitated by their ability to support CTLL-2 cell proliferation. While 4 of 10 individuals responded with IL-2 secretion (median 0.92 IU/ml) after stimulation with recombinant NSP4, 6 of 10 individuals secreted IL-2 (median 2.5 IU/ml) after stimulation with the $NSP4_{114–134}$ peptide (Table III). In contrast to the T-cell proliferation data, 4 of 6 individuals whose PBMCs produced IL-2 were positive with both NSP4 antigens.

Supernatants from antigen-stimulated PBMC were also tested for IFN-γ stimulation. Eight of 10 individuals secreted IFN-γ after stimulation with recombinant NSP4 (median 392 pg/ml) or after stimulation with the $NSP4_{114–134}$ peptide (median 291 pg/ml). Six of these eight individuals were positive with both NSP4 antigens. It is interesting to note that PBMCs from one individual (no.8), who was negative by T-cell proliferation and IL-2 assays, secreted IFN-γ in significant amounts after stimulation with both NSP4 antigens (Table III).

Computer HLA Peptide Motif Search

A computer HLA peptide motif search was performed to evaluate whether the $NSP4_{114–134}$ harbors T-cell epitopes with the capacity to induce a cytolytic T-cell response. Approximately 50% of the Swedish population contain HLA A2.01 molecules. The computer analysis showed that the best putative HLA-A2.01 binding region in the peptide was present at aa 115–124 KLTTREIEQV-sequence). In addition, the $NSP4_{114–134}$ peptide contains at least two binding motifs for HLA-B27 and HLA-A1 by computer HLA peptide motif search suggesting that this protein may contain MHC class binding motifs for several MHC types (data not shown).

DISCUSSION

While significant effort has been given to structural proteins of rotavirus (i.e., VP4, VP7), no or limited information is available concerning the humoral and cellular immune responses to nonstructural proteins. Considering that the nonstructural NSP4 protein of ro-

TABLE III. Cellular Immune Responses to rNSP4 and a Synthetic Peptide Corresponding to Residues 114–134 of NSP4 in Swedish Adult

	T-cell proliferation		IL-2 release		Interferon-γ release	
Individuals	rNSP4 (SI)[b]	$NSP4_{114–134}$ (SI)	rNSP4 (IU)	$NSP4_{114–134}$ (IU)	rNSP4 (pg/ml)	$NSP4_{114–134}$ (pg/ml)
1	7.5[a,b]	3.6[a]	0.20[a]	2.90[a]	245[a]	290[a]
2	2.5	15.2[a]	0.11[a]	0.90[a]	70[a]	205[a]
3	1.8	13.9[a]	0.15[a]	3.10[a]	120[a]	265[a]
4	6.9[a]	13.7[a]	3.20[a]	4.90[a]	585[a]	540[a]
5	1.2	7.3[a]	<0.05	2.80[a]	275[a]	<0.2
6	5.6[a]	1.5	<0.05	0.20[a]	535[a]	520[a]
7	7.3[a]	1.3	<0.05	<0.05	<0.2	19[a]
8	3.0	0.3	<0.05	<0.05	225[a]	200[a]
9	1.6	0.4	<0.05	<0.05	<0.2	<0.2
10	2.4	1.6	<0.05	<0.05	<0.2	<0.2

[a]Specific results.
[b]Stimulation index.

tavirus is capable of inducing diarrhea in infant mice and that antibodies directed against NSP4 can protect infant mice from diarrhea after challenge with infectious virus [Ball et al., 1996], humoral and/or cell-mediated immune responses against NSP4 may develop in humans after infection or vaccination. These immune responses possibly could participate in the recovery process after a rotavirus infection.

Ishida et al [1997] examined humoral immune responses by immunofluorescence to individual rotavirus proteins after heterologous and homologous infections in mice and found no appreciable immune responses to NSP4. This seems surprising because viral replication and expression of both structural and nonstructural proteins occur in adult mice given infectious murine (EPH_w strain) or rhesus rotavirus based on detection of virus antigen shedding, even though clinical diarrhea is not induced. By contrast, adult rabbits that also shed virus and do not develop diarrhea after infection with rotavirus, do exhibit antibody responses to NSP4 as detected by immunoprecipitation when the homologous antigen is used [Conner et al., 1988].

Similar to the findings of Richardson et al. [1993], we found that most individuals developed IgG antibody responses to NSP4 after infection. However, the titers were modest as compared with titers to VP6. Nicaraguan adults had higher IgG anti-NSP4 antibody titers than were found in Swedish adults, but the difference in titers was not statistically significant ($P > 0.5$). The fact that the Nicaraguan adults also had higher anti-VP6 titers than the Swedish adults might indicate that Nicaraguan adults are re-exposed more frequently to rotavirus than Swedish adults.

IgG immune responses to various polypeptides after natural rotavirus infection in children were previously examined. It was not possible to recognize specifically NSP4 antibodies in 8 of 8 children examined by immunoprecipitation [Svensson et al., 1987a], in contrast to the present study, in which NSP4 antibodies were found by ELISA in all naturally infected children. The discrepancies between these two observations could be attributable to the different methodologies used [RIPA vs. ELISA] or to significant antigenic differences between NSP4 from different rotavirus serotypes/strains. Support for the latter hypothesis is provided by Richardson et al. [1993], who found that the immune responses to NSP4 varied with the infecting serotype (G type). Serum antibody responses to NSP4 were seen in patients with serotype 1 (G1) rotavirus infection but detection of such responses varied at 67–100%, depending on the rotavirus strain used as antigen. Furthermore, NSP4 from a serotype G2 strain was not immunoprecipitated by any serum sample from patients infected with a serotype G4 virus, but was recognized by 67% of serum samples from serotype G1-infected patients. These results suggest that antigenic differences occur in NSP4 between different rotavirus strains/serotypes. Sequence analysis of NSP4 has revealed differences significant enough to classify NSP4 into three different groups [Cunliffe et al., 1997; Horie et al., 1997; Kirkwood and Palombo, 1997] with the greatest sequence variation occurring between residues 135-141 [Horie et al., 1997]. It is interesting to note that the RRV vaccine strain is highly divergent from other strains and forms one of five members of the third group that only contains G3 viruses [Cunliffe et al., 1997; Horie et al., 1997]. The implications of these findings for the use of RRV as a vaccine remain to be elucidated. However, it is of interest that G1 and G4 human rotavirus strains group together as subgroup II viruses while most G2 strains belong to subgroup I. Since the NSP4 groups correlate with subgroups of VP6 [Cunliffe et al., 1997], these results could explain the data from Richardson et al. [Richardsson et al., 1993].

This is the first study to specifically investigate humoral immune responses to NSP4 after rotavirus vaccination. The results indicate that three doses of the RRV-TV vaccine [Joensuu et al., 1997] induces a significant anti-VP6 immune response, but the anti-NSP4 response is modest. However, because the immunizing RRV NSP4 belongs to group III, and SA11 NSP4 was used as the antigen in our ELISAs, higher titers might be obtained if the same sera were tested with the homologous RRV antigen or peptide. This may explain the discrepancy found in seroconversion between NSP4

and VP6. It was interesting to observe that all but one of the protected children developed NSP4 antibodies (GMT 259 ± 176), but this occurred in only 1 of 3 (#12) of the nonprotected children. Unfortunately, the small numbers of available sera from the children in the nonprotected group does not allow for statistical analysis for a correlation between NSP4 antibodies and protection.

The IgA immune responses to NSP4 after rotavirus vaccination were examined for the first time and showed that while serum IgA anti-VP6 antibodies developed in 8 of 9 protected children, none developed detectable anti-NSP4 IgA antibodies. This suggests that serum IgA antibodies to NSP4 may not play a significant role in protection against rotavirus disease. Furthermore, because NSP4 is a nonstructural protein currently only known to be expressed within infected cells, it is unclear if mucosal IgA anti-NSP4 antibodies would play a role in protection against rotavirus infection. However, future studies should also examine mucosal anti-NSP4 responses.

To complement the immune response information obtained using rNSP4 protein, a synthetic peptide representing a portion of the proposed "toxin" region [Ball et al., 1996] of NSP4 (aa114-134) was synthesized and used in an IgG-ELISA. Fewer individuals responded with IgG antibodies against the peptide than against the recombinant protein ($P < 0.001$). The limited immune response against the peptide might be attributable to the fact that this region is hidden or partly hidden in the native protein, as immunizations with the peptide alone induce strong humoral antibody responses in animals (data not shown). Alternatively, antipeptide responses may need to be measured to peptides containing additional C-terminal residues since mutations associated with virus virulence have been reported at aa 135, 136, and 138 [Zhang et al., 1998], and these residues were absent in the peptide studied in this report.

Cell-mediated immune responses to NSP4 (recombinant and peptide) were detected in 8 of 10 Swedish adults and recorded as either a T-cell proliferative response or as an increased production of IL-2 or IFN-γ. These are the first observations showing that the NSP4 enterotoxin is capable of inducing a cellular immune response, while immunodominant T-cell epitopes were previously described on enteric toxins such as cholera toxin (CT-A and CT-B) [Cong et al., 1996]. A lymphoproliferative response to rotavirus antigens was previously described in adults [Totterdell et al., 1988]. In four of seven responders in the T-cell proliferative assay, the synthetic peptide showed a higher stimulation index than with the recombinant NSP4 protein. Also, a greater release of IL-2 was noted in cells stimulated with the synthetic peptide than with the recombinant protein. A reasonable explanation for this could be that rNSP4 may contain suppressive epitopes that abolish weak positive T-cell responses. If the peptide lacks these epitopes, this could explain why the peptide induced a greater cell-mediated response. Alternatively, the molar effectiveness of the peptide epitope was higher in the synthetic peptide preparation than in the whole recombinant protein preparation. It has previously been observed that peptides are more efficient T-cell stimulators than native proteins [Moore and Fox, 1993].

T cells are important for rotavirus clearance in both immunodeficient children [Wood et al., 1988] as well as in SCID mice and Rag 2–/– mice, who all become chronically infected [Franco and Greenberg, 1997; McNeal et al., 1997]. Furthermore, in the mouse model, cytotoxic T lymphocytes develop after a rotavirus infection and have been shown to resolve rotavirus infections, while the CTL response seems less important in protecting against reinfection [Offit et al., 1991; Franco and Greenberg, 1995; Ward, 1996; Rose et al., 1998]. The IL-2 and IFN-γ observations in this study suggest that NSP4 in humans is capable of inducing a Th1-like response [Powrie and Coffman, 1993]. It may be possible that a NSP4 peptide or full-length protein contains T-cell epitopes with the capacity to induce cytolytic T-cell responses. Cytotoxic T-cell epitopes have been identified so far in mice on VP3, VP5, VP6 and VP7 [Franco et al., 1993; Franco et al., 1994; Heath et al., 1997]. The cytokine-release observations together with HLA class I binding computer modeling indicate that the NSP4 peptide could bind to MHC class I pockets of HLA molecules or at least function as a Th1-type CD4 helper (epitope) determinant. Also the modest antibody titers to NSP4 suggest that NSP4 contains T-cell, rather than B-cell, epitopes. If a CTL response in humans against NSP4 can be identified, the clinical impact of such a response should be explored.

In conclusion, it was shown that NSP4 can induce a cellular immune response in humans, in addition to a humoral immune response after oral RRV-TV vaccination and natural rotavirus infection. This suggests that NSP4 epitopes bind to MHC class 1 pockets on HLA molecules. It is also suggested, based on indirect evidence from the IL-2 and IFN- data, that NSP4 induces a CTL response. These results indicate that larger studies of the immune response to NSP4 are needed, particularly by using homologous NSP4 antigens, but also by testing mucosal secretions in which there are indications of higher titers. It remains to be determined whether monitoring immune responses to NSP4 will provide the first clear correlate of protection against rotavirus infection.

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